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Dose Dependency of Aflatoxin B_1 Binding on Human High Molecular Weight DNA in The Activation of Proto-Oncogene

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The binding of aflatoxin B_1, AFB_1, a potent hepatocarcinogen, to various high molecular weight (HMW) DNAs from human normal liver and two liver cancer cell lines, Alexander primary liver carcinoma (PLC) and Mahlavu hepatocellular carcinoma (hHC) and from NIH/3T3 cell have been investigated. The kinetics of AFB_1 binding to these DNAs showed similar initial rates but the extents of binding to the PLC and hHC DNAs seemed to be slightly higher. Preferential AFB_1 bindings were identified in both PLC and hHC DNAs compared to normal liver DNA when analyzed by restriction endonuclease digestions and agarose gel electrophoresis. A critical AFB_1 binding dosage, ranging 100 to 460 fmole/μg DNA, was found to activate the carcinogenic effect of the Mahlavu hHC HMW DNA, but not normal liver HMW DNA, rendering it capable of inducing focal transformation in NIH/3T3 cell. Excessive AFB_1 binding on the hHC and PLC HMW DNAs resulted in an "over-kill" of both cell transformation capability and templating activity of the DNA.

Introduction

The highly carcinogenic agents are frequently also potent mutagens (*1*). Their insults are often directed at macromolecules such as DNA generating modifications by way of chemical interactions that result in lethal lesions or codon alteration(s) similar to (point) mutations. Aflatoxin B_1 (AFB_1), a metabolite of *Aspergillus flavus*, is well known for its potency in hepatocarcinogenesis in numerous animal species including trout, rat, hamster, dog, and rhesus monkey (*2*). AFB_1 has also been implicated in the etiology of human liver cancer on the basis of significant statistical correlations between the increased occurrence of liver cancer in areas of Asia and Africa and the consumption of grains contaminated with AFB_1 (*3–5*). Furthermore AFB_1 was reportedly detected in human liver cancer tissue samples (*5,6*). In the experimental animal, AFB_1 was found to be activated by microsomal mixed-function oxidases in liver cells to form AFB_1-2,3-epoxide (*7*), which then bound to the N^7 of guanine in both DNA and RNA leading to adduct formation (*7,8*). More recently, it has been reported that by using monoclonal antibody specific against AFB_1–DNA adduct, AFB_1–DNA adducts were detected in human urine samples and in liver biopsy samples obtained from patients (*9*). In fibroblast tissue culture AFB_1 has to be activated before it can act as a tumor initiator in conjunction with TPA, the tumor promotor, and is known to induce mutation in Chinese hamster cells and transform C3H/10T1/2 mouse fibroblasts (*10*). The ability of AFB_1 to react with the cell's DNA in adduct formation thus depends on the activation of AFB_1 to its reactive form by the cellular microsomal peroxidase such as in the liver cell. In order to understand the molecular mechanism in which AFB_1 induces the process of hepatocarcinogenesis, it requires precise analysis on the binding affinity of AFB_1 with human liver DNA, the possibility of preferential targets within the genomic DNA as well as the genomic nature of the targeted DNA. In this investigation we have examined the formation of adducts between ^3H-labeled AFB_1 with several human DNA samples derived from Alexander primary hepatocellular carcinoma (PLC), Mahlavu hepatocellular carcinoma (hHC), and a normal adult liver sample by agarose gel electrophoresis.

The DNA fragments, preferentially targeted by AFB_1

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were also defined by restriction endonuclease analysis. The biological activity of AFB_1-bound high molecular weight (HMW) DNAs with respect to (1) DNA-mediated cell transformation by transfection and (2) the templating efficiency of the AFB_1-bound DNAs in DNA polymerase assay was also analyzed.

Materials and Methods

Tissues and Cell Cultures

A normal adult liver (NAL) from a young adult was fresh tissue kindly provided by Dr. A. Huang, Fairfax Hospital, VA. (Alexander) PLC is human primary hepatocellular carcinoma currently maintained in tissue culture. It carries the hepatitis B marker. MAH, Mahlavu hepatocellular carcinoma, is a tissue culture cell line recently established from a hepatocellular carcinoma obtained from an African patient. NIH/3T3 is a mouse fibroblast cell line kindly provided by Dr. D. Lowy of the National Cancer Institute (Bethesda, MD). Cells in culture are maintained in Dulbecco modified Eagle's media supplemented with 10% fetal calf serum (56°C, 30 min) plus penicillin (50 units/mL) and streptomycin (25 μg/mL) in a 5% CO_2 atmosphere at 37°C. All tissue culture materials were purchased from GIBCO (Grand Island, NY), and Microbiological Associates (Bethesda, MD).

DNA Preparation and AFB_1 Binding Reaction

Total and high molecular weight DNA was prepared from fresh liver tissue, minced and homogenized, or from frozen liver after pulverization in liquid N_2 or from tissue culture cells in lysing buffer containing proteinase K (final concentration, 75 μg/mL), 50 mM Tris-HCl, pH 8.1, 1 mM EDTA, and 1% SDS, at 37°C for 2 hr as described earlier (*11*). The preparations were then purified by sequential phenol–cresol, chloroform–isoamyl alcohol, and ether extractions followed by ethanol–NaCl precipitation at -20°C overnight. The DNA samples were then further purified by RNase digestion followed by a second sequence of phenol–cresol, chloroform–isoamyl alcohol, and ether extractions and ethanol–NaCl precipitation. When needed, the DNA samples were further banded by cesium chloride gradient centrifugation at 50,000 rpm for 18 hrs. The DNA samples were then dialyzed against Tris-HCl, 20 mM, EDTA, 1 mM, and NaCl, 0.1 M (TEN buffer) exhaustively prior to their use in experiments.

$[^3H]AFB_1$ was purchased from Morovak Laboratory, CA, at 15 Ci/mmole. It was further purified by HPLC and the resultant single peak of $[^3H]$-AFB_1 had the specific activity of 9250 cpm/pmole in chloroform. The procedure for $[^3H]AFB_1$ binding to DNA was as described earlier (*8,12*). Briefly, $[^3H]AFB_1$ was first activated either by incubation with microsomal oxidase preparation or chemically with perchlorobenzoic acid and methylene chloride to the epoxide form prior to its interaction with DNA. Kinetics of the $[^3H]AFB_1$–DNA adduct formation was determined by withdrawing 25 μL samples at specified intervals and precipitating onto nitrocellulose filters in 10% trichloracetic acid at 0°C, followed by 10 washes of each filter with chloroform, 1.5 mL each time. The filters bearing the $[^3H]AFB_1$–DNA adduct were dried and counted by a liquid scintillation method. Simultaneously 250 μL $[^3H]AFB_1$–DNA samples were withdrawn and purified by alcohol precipitation until no free $[^3H]AFB_1$–DNA can be detected. They were further extensively washed with chloroform prior to their use in analyses by agarose gel electrophoresis after restriction enzyme digestions, or by DNA-mediated cell-transformation assays.

Agarose Gel Electrophoresis and Restriction Endonuclease Analysis of $[^3H]AFB_1$–DNA

Purified $[^3H]$-AFB_1–DNA was analyzed by agarose gel electrophoresis with or without digestions with the following restriction endonucleases: Eco RI, Bam HI, Hind III, Pst I, Kpn I, Alu I, Hae III, and Taq I. The conditions for the assay reactions of the numerous restriction endonucleases were as described in the procedures provided by the various laboratories (New England Biolab, Beverly, MA; International Biotechnology Institute, New Haven, CT; Bethesda Research Laboratory, Rockville, MD). All reactions were carried to completion. The $[^3H]AFB_1$–DNA fragments were purified by phenol extraction and a rapid ethanol precipitation at 0°C prior to analysis by agarose gel electrophoresis. Agarose gel electrophoresis was carried out with 1% agarose at 50 V for 6 hr as described (*11*). The gel was scanned for relative DNA concentration by absorbency at 260 mμ. It was then fractionated into 40 to 50 samples and the radioactivity determined. In some experiments the agarose gel was treated in Enhance (New England Nuclear, Boston, MA), rehydrated, dried, and fluorography taken. Recovery of DNA from agarose gel was carried out by electroelution (*11*).

Cell-Transformation Assay by DNA Transfection

Transfection of NIH/3T3 cells with purified HMW DNA was carried out as described elsewhere (*13*). Briefly, human HMW DNAs, isolated and purified from Mahlavu hepatocellular carcinoma cells (hHC DNA), and other HMW DNAs prepared from other sources, such as NIH/3T3 cells and normal adult liver, with or without $[H^3]AFB_1$ activation at varying doses, were used to transfect 10^5 NIH/3T3 cells. After 24 hr, the transfected culture was trypzinized and divided into two plates. As an option TPA, a tumor promoting agent, was added to one of the two daughter cultures at optimal concentration, 0.1 μg/mL, for 3 days. The cultures were fed every 3 days. Scoring of cell transformation was carried out

on day 9, 21, and 30 for focus forming unit. Transformed foci were cloned out by penny-cylinder trypsinization. The DNAs were extracted and purified from each transformed clone or from transfected cultures for Southern blot hybridization against [^{32}P] nick-translated HMW hHC DNA for verification of the integration of the hHC DNA as described below.

DNA–DNA Hybridization Analysis and Preparation of [^{32}P] Nick-Translated hHC DNA Probe

High molecular weight DNAs extracted and purified from various cloned, transformed cells were digested with numerous restriction endonucleases and analyzed in 0.8% or 1.0% agarose gel electrophoresis. DNA samples in the gel was transferred onto nitrocellulose filter (Schleicher & Schuell, Keene, NH) by the Southern blot method (*11*). Filter hybridization was carried out as described earlier (*11*) in stringent conditions, with [^{32}P] nick-translated human HMW DNA at 10^6 cpm/mL in Deinhardt solution at 37°C for 24 hr. The filter was washed stringently and exhaustively prior to fluorography. Mahlavu hHC DNA was extensively purified as described above and then subjected to Hind III digestion for 2 hr prior to nick-translation with [^{32}P]dGTP (3000 Ci/mm; Amersham, Arlington Hts., IL), three other cold deoxynucleoside triphosphates, *E. coli* Polymerase I, and DNase (*11*). The [^{32}P]-hHC DNA was then precipitated, washed, redissolved in 1/10 Ten buffer, and dialyzed extensively prior to its use as a probe.

Tumorigenesis

Tumorigenicity of the cloned transformed cells and other transfected cultures that showed no transformed foci were analyzed by inoculating 10^6 viable cells into nude mice. Secondary passage of tumor was carried out in both NIH Swiss and Balb/c mice for further verification. DNAs were also prepared from each tumor for Southern blot hybridization against [^{32}P] nick–translated HMW hHC DNA.

DNA Polymerase Assay

DNA polymerase assay was carried out with *E. coli* polymerase I. The assay condition was as described in the procedure provided by the supplier (Bethesda Research Laboratory, Rockville, MD). Briefly, cold dATP, dGTP, TTP (at a final concentration of 10^{-4} M) and 10 μCi of [^{32}P]dCTP (3000 Ci/mm; Amersham, Arlington Hts., IL) were used in 100 μL assay volume containing 5 μg of template DNA. At specified times 5 μL sample was withdrawn, precipitated in 10% trichloroacetic acid (TCA) on nitrocellulose filter at 0°C. The filter was washed extensively with 10,000 volumes of cold 5% TCA, then with 80% ethanol; it was then dried and counted by a liquid scintillation method.

Results

Kinetics of [^{3}H]AFB_1 Binding to HMW DNAs

Aflatoxin B_1 binds efficiently to all the HMW DNAs tested. Figure 1 shows the kinetics of [^{3}H]AFB_1 binding

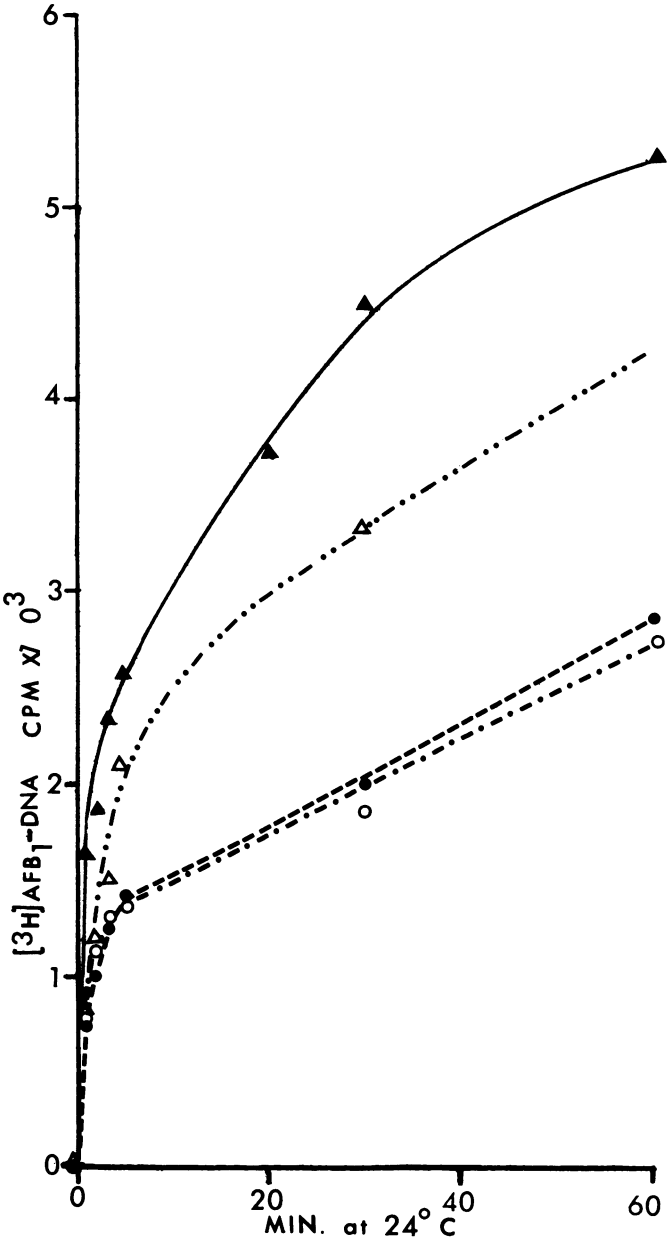

FIGURE 1. Kinetics of [^{3}H]AFB_1 binding to various high molecular weight DNAs prepared from (●) normal adult liver; (Δ) primary carcinoma cell (PLC); (▲) Mahlavu hepatocellular carcinoma cell (hHC); (○) nonhuman DNA from NIH/3T3 mouse fibroblasts. [^{3}H]AFB_1 was activated and bound to HMW DNA as described in the text in a final volume of 1.0 mL. Aliquots, 5 μL containing 1.0 μg of HMW DNA were withdrawn and TCA precipitated on nitrocellulose filter. Samples were washed extensively with 10,000 volumes of ice-cold chloroform. The amount of [^{3}H]AFB_1 bound to DNA on the filter was then determined by a liquid scintillation method (*11*).

to human HMW DNAs prepared from a normal adult liver, primary carcinoma cell (PLC), and Mahlavu hepatocellular carcinoma cell (hHC). Binding to one nonhuman DNA obtained from NIH/3T3 mouse fibroblasts was also analyzed. It is apparent that the initial rates of AFB_1 binding to all these DNAs were extremely rapid and linear although the extent of AFB_1 binding might be slightly higher with PLC and Mahlavu hHC DNAs.

Preferential Binding of Subgenomic DNA Fragments by [^{3}H]AFB_1

High molecular weight DNAs prepared from normal adult liver and from Mahlavu hepatocellular carcinoma cells, bound to [^{3}H]AFB_1 at saturation level, were exhaustively washed free of unbound AFB_1 with chloroform prior to ethanol precipitation. The [^{3}H]AFB_1–DNAs were subjected to various restriction endonuclease digestions. Among the numerous restriction endonucleases tested on the human HMW DNAs in this study which included Eco RI, Bam HI, Hind III, Pst I, Kpn I, Alu I, Hae III, and Taq I, Hind III restriction yielded the most informative pattern. Figure 2 shows the profile of the Hind III restricted [^{3}H]AFB_1–DNAs of normal adult liver as compared to that of Mahlavu hHC HMW DNA when analyzed in agarose gel electrophoresis. Taking the DNA concentration at A_{260} into consideration, preferential binding of the Hind III restricted hHC subgenomic DNA fragments by [^{3}H]AFB_1 are evident at the following peaks: (1) >12 kb; (2) 2.6–3.2 kb; (3) 1.4–1.8 kb; (4) 500–650 bp; (5) 350 bp; and (6) 50–100 bp, which showed considerably high specific activities of [^{3}H]AFB_1cpm/μg of DNA. Normal adult liver DNA showed most binding at the high molecular weight band, > 12 kb, with little binding at the 1.4–3.2 kb regions. Considerable binding at the 50–100 bp, 350 bp, and 500–650 bp was seen in both samples. These represented the human repetitive Alu I/Hae III sequence when analyzed by hybridization against the [^{32}P] nick-translated Alu I/Hae III human DNA (*14*) which is rich in GC contents (data not shown).

Dose Effect of [^{3}H]AFB_1 Binding in the Activation of a Human Proto-Oncogene

It has recently been reported that chemical carcinogen could activate a proto-oncogene and render it capable of cell transformation when assayed by the DNA-mediated cell transformation assay in suitable indicator cells (*15*). We had also examined the capability of the AFB_1-bound HMW DNA prepared from Mahlavu hepatocellular carcinoma cells at various [^{3}H]AFB_1 concentrations to transform NIH/3T3 cells. As controls, we chose normal adult liver DNA and NIH/3T3 cell DNA, with or without [^{3}H]AFB_1 binding. Table 1 summarizes the [^{3}H]AFB_1 binding to the various DNAs at increasing concentrations and the number of transformed foci per 10 μg of [^{3}H]AFB_1–DNA obtained in the DNA-mediated cell transformation assay. AFB_1 bound all tested

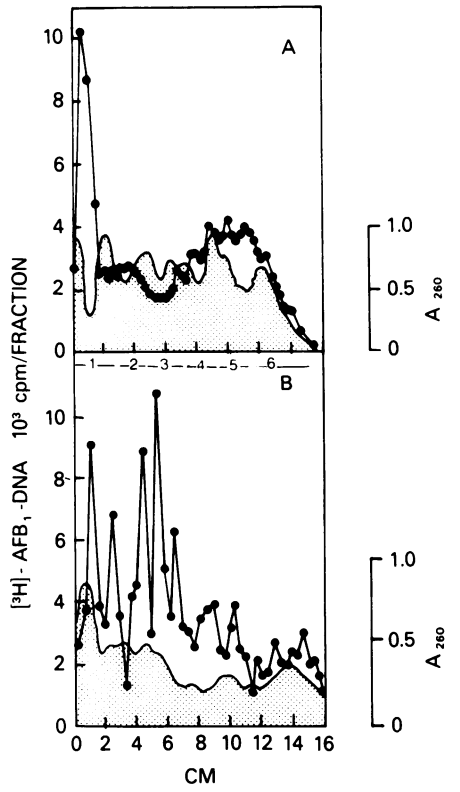

FIGURE 2. Electrophoretic patterns of [^{3}H]AFB_1-bound DNAs prepared from normal adult liver and from Mahlavu hepatocellular carcinoma cell. Approximately 20 μg of [^{3}H]AFB_1–DNA was digested with Hind III restriction endonuclease prior to its analysis in agarose gel (1.0%) electrophoresis. Details of all procedures were as described in "Materials and Methods."

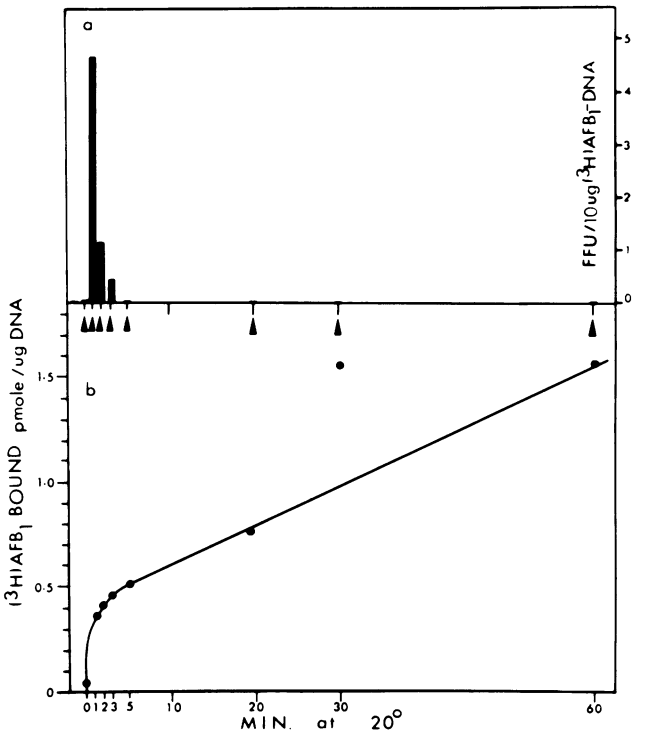

FIGURE 3. Dose dependency of [^{3}H]AFB_1 activation of Mahlavu hepatocellular carcinoma DNA in DNA-mediated cell transformation study. All experimental procedures were as described in Table 1.

HMW DNAs prepared from normal adult liver (NAL), Mahlavu hHC, and NIH/3T3 cells equally well. However, it is evident that, within a narrow dosage range, AFB_1 activated only the HMW DNA from Mahlavu hHC cells and rendered the hHC HMW DNA capable of transforming NIH/3T3 cells in the transfection assay (Fig. 3). These critical doses range from 100 to 460 fmole/μg HMW DNA. In contrast, AFB_1 binding did not render the HMW DNA from the other sources (NAL and NIH/3T3) capable of cell transformation.

The addition of TPA to the transfected NIH/3T3 cultures did not increase the number of transformed foci in the assay (Table 1). On the other hand, it seemed to augment the carcinogenic effect of the mutagenic AFB_1 bound hHC DNA in the transfection assay. Focal transformation appeared earlier by 2 to 3 days in the presence of TPA, and the foci appeared larger and more prominent. Similar to observations reported elsewhere (*10,16*), TPA was thus considered a promotor and not an initiator in this study.

The transformed cells were successfully cloned by penny-cylinder, and DNA was extracted from the transfected clones for verifying the integration of the hHC AFB_1–DNA in the mouse cell's genome. Figure 4 shows the Southern blot hybridization of the purified transfectant DNAs from various transformed clones and from other transfected cultures, that showed no transformation, against [^{32}P] nick-translated Mahlavu hHC DNA digested by Hind III restriction endonuclease. Clones 1 and 2 showed 4.8 and 1.2 focus-forming units (FFU) per 10 μg of the transfectant DNA hybridization with the [^{32}P] hHC DNA probe, integrated within the NIH/3T3 mouse cell DNA. Other negative cultures of NIH/3T3 cells that did not exhibit any focal transformation at the end of the transfection assay were also cultured out for DNA isolation for hybridization analysis. As expected, no human DNA was detected in clones 3 transfected by [^{3}H]AFB_1–DNA of NIH/3T3 cells. The negative cultures (4, 5, 6) transfected with [^{3}H]AFB_1 activated hHC DNAs at high doses of AFB_1, however, did show positive integration of hHC DNA when probed with the [^{32}P]-labeled hHC DNA restricted with Hind III. The human DNAs, however, did not show identifiable bands as in clones 1 and 2, but rather diffused patterns. The transformed phenotype was thus most probably conferred by the exogenous human [^{3}H]AFB_1–DNA activated at an optimal dosage without disruption of the molecular integrity of the hHC DNA in the recipient NIH/3T3 mouse cells. We further examined if the exogenous human DNA was stably integrated into the mouse cell DNA by a secondary transfection assay using the transfectant DNA on NIH/3T3 cells. Results (not shown) showed an increase in the efficiency of the tranfectant DNA in transforming NIH/3T3 cells.

Tumorigenicity of the Transformed NIH/3T3 Cells Transfected with [^{3}H]AFB_1–DNA of Mahlavu Hepatocellular Carcinoma Cell

The tumorigenicity of the transformed NIH/3T3 cells

Table 1. Activation of carcinogenic activity of human high molecular weight DNA by binding with aflatoxin B_1.[a]

Sources of HMW DNA	[^{3}H]AFB_1 bound, fmole/μg DNA	DNA-mediated focal transformation, FFU/10 μg of [^{3}H]AFB_1–DNA	
		No TPA	Plus TPA
NIH/3T3 cell	0	0	0
	123	0	0
	252	0	0
	395	0	0
	682	0	0
Human normal liver	0	0	0
	112	0	0
	210	0	0
	392	0	0
	455	0	0
Mahlavu hepatocellular carcinoma cell	0	0	0
	197	4.8	4.1
	248	0.9	1.0
	273	0.4	0.2
	403	0.1	0
	843	0	0
	867	0	0

[a] [^{3}H]AFB_1 was activated and bound to HMW DNA as described in the text in a final volume of 1.0 mL. Aliquots, 5 μL containing 1.0 μg of HMW DNA, were withdrawn and TCA precipitated on nitrocellulose filter. Samples were washed extensively with 10,000 volumes of ice-cold chloroform. The amount of [^{3}H]AFB_1 bound to DNA on the filter was then determined by a liquid scintillation method (*11*). Simultaneous samples of 50 μL each, containing 10 μg of HMW DNA, were withdrawn and precipitated in ethanol–NaCl. The [^{3}H]AFB_1–DNA in these samples were prepared and used in cell transformation study by DNA transfection assay as described in "Materials and Methods."

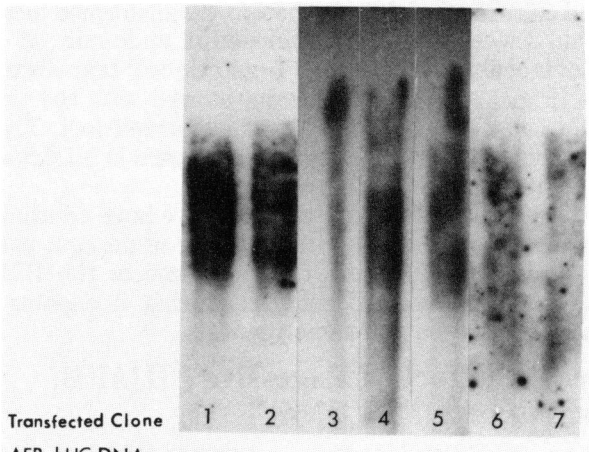

FIGURE 4. Detection of human HMW DNA in transformed clones and in transfected, nontransformed cultures of NIH/3T3 cell by Southern blot, DNA–DNA hybridization analysis. DNAs were isolated from the transformed clones and from transfected, nontransformed cultures of NIH/3T3 cell. Approximately 10 μg of DNA was used in each sample for hybridization. Approximately 2×10^6 cpm of [^{32}P]-nick-translated hHC DNA per filter was used in hybridization. All experimental procedures and conditions were as described in "Materials and Methods."

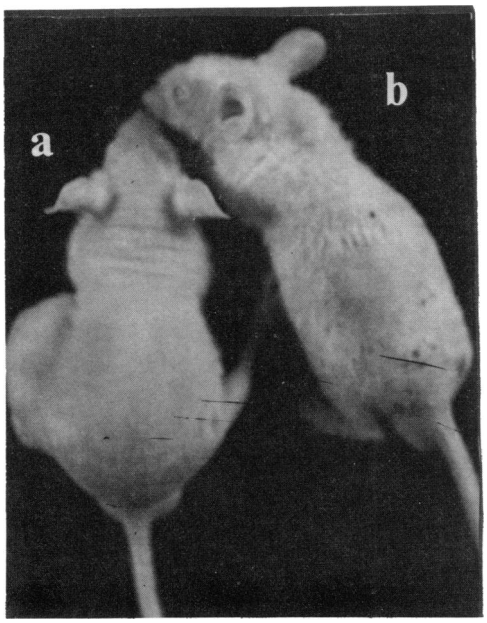

FIGURE 5. Tumorigenesis of transformed NIH/3T3 cells transfected by Mahlavu hepatocellular carcinoma DNA in nude mice: (*a*))tumor developed in nude mouse inoculated with 10^6 transformed NIH/3T3cells transfected with Mahlavu hHC [^{3}H]AFB$_1$–DNA; (*b*) nude mouse inoculated with 10^6 transfected NIH/3T3 cells transfected with [^{3}H]AFB$_1$–DNA of NIH/3T3. All procedures were as described in "Materials and Methods."

transfected by the [^{3}H]AFB$_1$–hHC DNA was also examined by injecting 10^6 transformed cells each from clone 1 and clone 2 into NIH Swiss nude mice. Similarly, a tumorigenicity test was also carried out with transfected cultures that did not show any transformed focus. Within 3 weeks a tumor developed in nude mice at the site of inoculation with clone 1- and clone 2-transformed cells (Fig. 5) but not in mice inoculated with the tranfected cells which showed no transformed foci. These tumors were eventually successfully passed in NIH Swiss mice but not in Balb/c mice.

On the basis of these observations we have concluded that aflatoxin B$_1$, at an optimal range of dosage, probably activated a human proto-oncogene in the HMW DNA of Mahlavu hHC cells, rendering it capable of transforming cells and tumorigenesis.

Overkill Effect of Excessive [^{3}H]AFB$_1$ Binding on HMW DNA

Excessive AFB$_1$ binding on HMW hHC DNA abolished all biological transformation capability such as observed in samples with greater than 450 fmole of AFB$_1$ bound/μg of the HMW hHC DNA (Fig. 3). In the four samples with binding of AFB$_1$ at 520, 745, 1550, and 1600 fmole/μg of HMW hHC DNA, no transformed foci were observed, although human HMW DNA was apparently detectable in these cultures when examined by DNA–DNA hybridization analysis as discussed above (Fig. 4). When the biochemical parameter of templating capability of the DNAs bound with AFB$_1$ at varying doses was examined, an inverse relationship seemed to exist between increasing dosage of AFB$_1$ bound and the templating activity of AFB$_1$–DNA. Figure 6 shows a progressive loss of templating capability of the HMW DNA with increasing binding by [^{3}H]AFB$_1$ in DNA polymerase assays using *E. coli* DNA polymerase. We also obtained the same results in RNA polymerase assays (data not shown). When the products synthesized in the DNA polymerase reaction, using [^{3}H]AFB$_1$–DNA at 1462 fmole/μg DNA as template, were examined, short and heterogeneous fragments ranging from 20 to 100 nucleotides were obtained when determined by polyacrylamide gel (4%) electrophoresis (data not shown). Excessive binding of the AFB$_1$ on HMW DNA thus impaired the templating capability of the HMW DNA. AFB$_1$ covalently linked to the N^7 of the guanine of the DNA seemed to have caused the newly synthesized polynucleotide strand to terminate prematurely, so that only short polynucleotide products were synthesized in the DNA polymerase reaction.

Discussion

Other reports (*7,8,12,17*) and our current investigation demonstrated that aflatoxin B$_1$, when activated chemically or by microsomal oxidases to become the AFB$_1$-2,3-epoxide, tends to bind DNA efficiently. In our binding studies, [^{3}H]AFB$_1$ bound hepatocellular carcinoma DNA, digested with Hind III, at a differential pattern than that of young adult liver DNA. Preferential binding was observed with the hepatocellular car-

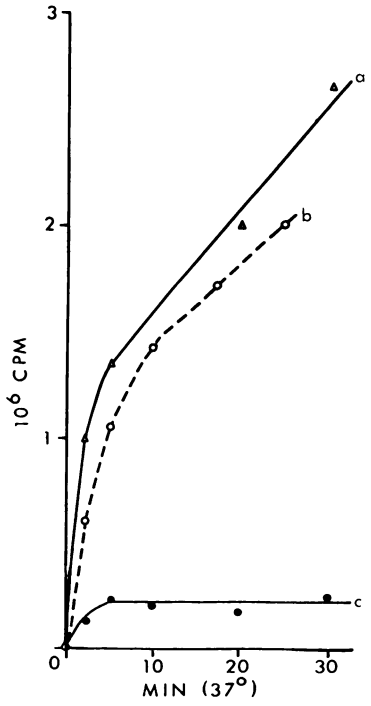

FIGURE 6. Template activity of [^{3}H]AFB$_1$–bound DNA of primary liver carcinoma (Alexander) cell: (*a*) PLC DNA with no aflatoxin binding; (*b*) PLC DNA bound with 152 fmole of [^{3}H]AFB$_1$; (*c*) PLC DNA bound with 3100 fmole of [^{3}H]AFB$_1$. DNA polymerase assay was as described in Methods. A 5-μL sample of [^{32}P]dCTP labeled product was precipitated in 10% ice-cold TCA on nitrocellulose filter. The filter was washed extensively and counted by a liquid scintillation method as described in the "Materials and Methods."

cinoma DNA at two subgenomic, Hind III restricted DNA fragments: 1.4 to 1.8 kb and 2.6 to 3.2 kb (Fig. 2). In contrast, normal adult liver DNA showed little or no binding by [^{3}H]AFB_1 in DNA fragments of similar sizes when examined in the same experiment. Similar [^{3}H]AFB_1 preferential binding patterns were seen in both hepatocellular carcinoma DNA as well as young normal liver DNA at subgenomic DNA fragments of 50 to 150 bp, 300 to 350 bp, and 500 to 650 bp. These probably corresponded to the human Alu I/Hae III repetitive sequences which are rich in GC contents (data not shown).

The binding of [^{3}H]AFB_1 on Mahlavu HMW DNA at optimal dosage of 100 to 460 fmole/μg DNA activated a proto-oncogene in the hHC HMW DNA, which demonstrated cell-transformation capability in transfection assay on NIH/3T3 cells (Fig. 3). It should be mentioned that, in the current study, total high molecular weight DNA (> 25 kb) was used in all experiments; thus the optimal AFB_1 binding dosage may be much higher than the optimal dosage required for a molecularly cloned proto-oncogene of molecular size of a few kilobase pairs. It will be especially meaningful to determine the optimal dosage necessary for AFB_1 activation of a human proto-oncogene with a known molecular size.

Excessive binding by [^{3}H]AFB_1 on Mahlavu HMW hHC DNA not only failed to activate the proto-oncogene but also diminished the template capability of the HMW DNA. In short an "overkill" effect in both DNA-mediated cell transformation and DNA templating capability was seen with excessive binding of AFB_1 on HMW DNA.

It has been well documented that aflatoxin B_1 is a potent hepatocarcinogen (*1,3,4*). Its mechanism of action has yet to be well understood. Our current study and others suggested that one possible mode of aflatoxin B_1 action resides in its affinity to form covalent linkage with the N^7 of guanine in high molecular weight DNA. As discussed above, preferential targets within the high molecular weight DNA were evident in our analysis with the Hind III digested [^{3}H]AFB_1–DNA from Mahlavu hHC cell. Others have suggested in their reports (*18,19*)— and our results in preliminary examinations on the nucleotide sequences targeted by AFB_1— indicated that AFB_1 attack on guanine is greatly affected by the vicinal nucleotide sequence. Among the preferred AFB_1 targets, we have obtained a 3.1 kb Hind III restriction fragment of Mahlavu hHC DNA, in which, we have established a Nar I restriction endonuclease site reading GCCGGC. The Nar I restriction endonuclease recognition sequence has been considered a diagnostic sequence for the 12th amino acid (GGC), glycine, of the n-ras oncogene family (*20*). In bladder carcinoma such a sequence has been mutated to GCCGTC, and thus the 12th amino acid was replaced by valine (GTC) (*20*). In our nucleotide sequence analysis, as well as suggested by others (*18*), AFB_1 shows high affinity with the second G in a tetranucleotide of CGGC. AFB_1 bound to the N^7 of guanine was reported to render the AFB_1-G incapable of base-pairing with C but rather mispairing with A thus resulting in a (G-C to A-T) transversion type of mutation (*21*). Whether a similar event has occurred in the HMW DNA of Mahlavu hepatocellular carcinoma cell upon AFB_1 binding at optimal concentration, awaits further detailed nucleotide sequence analysis.

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